Metabolites of endophytic fungi from Australian native plants as potential anticancer agents

Bita Zaferanloo[1], Stephanie A. Pepper[2], Sally A. Coulthard[2], Christopher P.F. Redfern[2], Enzo A. Palombo[1]

[1] Department of Chemistry & Biotechnology, School of Science, Faculty of Science, Engineering and Technology, Swinburne University of Technology, Hawthorn, Australia
bzaferanloo@swin.edu.au

[2] The Newcastle Cancer Centre, Northern Institute for Cancer Research, Newcastle University, Newcastle, United Kingdom

Abstract

Interest in endophytes as natural sources for new medicines was inspired by the discovery of paclitaxel-producing endophytic fungi. This study investigated the anti-cancer activity of extracts of endophytes isolated from two Australian plants, *Eremophila longifolia* (EL) and *Eremophila maculata* (EM). Endophytes were isolated from surface-sterilised leaf tissue, grown as pure cultures and identified by sequencing of ITS regions of the ribosomal DNA. To determine cytotoxicity, two leukaemic (MOLT-4, T-cell leukaemia; PreB-697, Pre-B leukaemia), a lung cancer cell line (A549) and a normal humans fibroblast cell line were treated with endophyte extracts to assess cytotoxicity in relation to alternariol monomethyl ether (AME) and alternariol (AOH). Endophyte extracts that showed cell cytotoxicity were analysed by UV HPLC to determine the metabolites. Pure AME and AOH, three extracts form *Alternaria* sp. (EM-6, EM-7 and EM-9) and one from *Preussia minima* sp. (EL-14) were cytotoxic to the cancer cell lines. All cytotoxic endophytes contained AME and AOH, the most cytotoxic endophyte EM-6 also contained two unique peaks. These data indicate that these four endophyte extracts may have anti-cancer properties due to the presence of AME and AOH, however the unique compounds found in the EM-6 extract may be exclusively cytotoxic and warrant further investigation.

Keywords: Metabolomics, Endophytic fungi, Anti-cancer agents, Australian native plants

Introduction

The discovery of effective anti-cancer agents from plants can be guided by the use of relevant plants in traditional medicine. Some of the first plant-derived natural products introduced into modern day clinical practice were the alkaloids, vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don. (Guéritte and Fahy, 2005). In spite of major investment in the development of new synthetic anti-cancer agents, the number of novel chemical compounds is declining and, as a consequence, there is now a systematic search for organisms, compounds and genes from the environment that might have potential biotechnological benefit as well as leading to new product development. Indeed, in the area of cancer, of the 175 small molecules identified between the 1940s to the end of 2014, 131 (75%) are other than "S" (synthetic), with 85 (49%) being either natural products or directly derived from them (Cragg and Newman, 2013, Newman and Cragg, 2016).

A more recent development is the investigation of endophytes as a potential natural source of drugs (Gutierrez et al., 2012). The relationships between endophytes and their host plants can vary from symbiotic to

pathogenic with some endophytic fungi isolated from plants found to produce important anti-cancer agents. Some of the most significant include Taxol (Stierle et al., 1993, Kharwar et al., 2014), camptothecin (Amna et al., 2006, Shweta et al., 2010), vinblastine (Okouneva et al., 2003), vincristine (Yang et al., 2003), Cercosporamide (Wang et al., 2012) and alternariol (Singh et al., 2014). Alternariol (AOH) is a toxic metabolite and one of the major secondary metabolites produced by various species of *Alternaria*. It is an important contaminant in cereals and fruits and also exhibits antifungal and phytotoxic activity which is reported to inhibit cholinesterase enzymes (Singh et al., 2014).The discovery and isolation of such metabolites should allow the development of processes to increase their production providing, of course, that the gene/gene product controlling their production by the relevant endophytes can be identified (Cragg et al., 2009).

The overall aim of this study was to determine if endophytes comprising a collection isolated from two native Australian plant species, *Eremophila longifolia* (EL) and *Eremophila maculata* (EM) (Zaferanloo et al., 2013), demonstrated cytotoxic activity on human cancer and normal fibroblast cell lines. Cytotoxicity of endophyte extracts and pure alternariol monomethyl ether (AME) and alternariol (AOH) (Fehr et al., 2009) was assessed using two human leukaemic cell lines, MOLT-4 and Pre-B 697, one lung cancer; A549 and a human fibroblast cell line. Active extracts were tested for the presence of known cytotoxic components.

Material and Methods

Isolation and identification of endophytes

Frozen leaves of *E. longifolia* and *E. maculata* were obtained from Canopus Corporation (Byrock, NSW, Australia) and a native plant nursery in Horsham (Victoria, Australia), respectively. The samples were isolated from surface-sterilised leaf tissue as described by Strobel and Daisy (2003) and grown as pure endophytic cultures on potato dextrose agar (PDA) plates for 5 days of growth at 25 ˚C. Extraction of fungal DNA was carried out using the ZR Fungal/Bacterial DNA kit (Zymo Research Corp). After assessing the quantity and quality of the extracted DNA, amplification and direct sequencing of fungal ITS regions were performed. The ITS1, 5.8S and ITS2 regions of the fungal DNA were amplified by PCR using the BioMix Red PCR kit (Bioline). The reaction mixture consisted of 10 µmol of each primer ITS1 (5'-TCC GAT GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The purified PCR products were sequenced and analysed using BLASTN software available at the National Center of Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to determine the identity of the endophytes (Huang et al., 2009, Zaferanloo et al., 2013).

Comparison of the ITS regions of endophytic fungi

To reveal evolutionary relationships between isolates, DNA sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/clustalw/) as described by (Zaferanloo et al. (2013)). The phylogenetic relationships of the isolates were evaluated using neighbour-joining analysis in MEGA version 5 (Tamura et al., 2011) with a bootstrap value obtained from 1000 replicates. Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). A bootstrap score of 1000 with values of greater than 50% indicated that the sequences shared significant homology.

Preparation of endophyte extracts

All fungal isolates were grown in Potato Dextrose Broth (PDB) and then filtered by membrane filtration through a 0.22 μm filter (Millipore) under vacuum to separate mycelium from broth (Xu et al., 2008). Each sterilized broth was frozen in autoclaved round-bottom flask for freeze-drying. The weight of the lyophilized extract was measured by first weighing the empty round-bottom flask before adding the filtrate and again after freeze-drying. The weight of the freeze-dried compound was calculated by the difference between the two weights and recorded. After freeze-drying, the lyophilized powder was mixed in sterile water to the same concentration (100 mg /mL) for all samples, kept at 4 °C and used to determine cytotoxic activity.

For cell cytotoxicity assays, 10 mg of each individual aqueous endophyte extract, alternariol monomethyl ether (AME) or alternariol (AOH), was in dissolved in 1 mL of Phosphate Buffered Saline (PBS) filtered through a 0.2μm Nalgene syringe filter (Thermo Scientific) and diluted to required concentrations in RPMI 1640 medium supplemented with 10% foetal calf serum (culture medium).

Cell culture and cytotoxicity assays

All cell lines were grown in RPMI 1640 medium supplemented with 10% foetal calf serum at 37 ^{0} C, 5% $C0_2$. For cytotoxicity assays, PreB697 and MOLT-4 suspension cells were seeded into 96-well plates at densities of 1.4 x 10^6/mL and adherent A549 lung cancer and primary fibroblast cells were seeded at densities of 1.5x10^4/mL and 3.5 x10^5/mL, respectively, and incubated overnight before addition of endophyte extract serially diluted to final concentrations ranging from 250.0 to 0.11 μg/mL, or serial dilutions of AME or AOH. Cells were then incubated for 72 hours (approximately three cell doublings to ensure detection of response) and cytotoxicity was assessed using the colorimetric CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) (MTS) following the manufacturer's protocol and absorbance measured at 490 nm.

MOLT4 and A549 human cell lines were purchased from American Type Culture Collection (ATCC); MOLT4 cells were derived from a patient with acute lymphoblastic leukaemia, and A549 cells were derived from a lung adenocarcinoma. . PreB-697 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and were derived from a human B cell precursor leukaemia at acute lymphoblastic relapse.

UV-HPLC

Those endophyte extracts that showed substantial cytotoxicity (>70% cell death at the highest concentration) in any of the cell lines were screened using UV-HPLC for the presence of AME and AOH (Fehr et al., 2009) and any other metabolites. AME and AOH standards were initially made to 1 mg/mL in methanol and further diluted to 10 μg/ mL in 50 %acetonitrile: 50% of 5% trifluoroacetic acid (TFA) in MilliQ water (w/w) (mobile phase) prior to injection. Endophyte extracts of those that elicited significant cytotoxicity >70%; EM6, EM7 and EM9 and EL-14 were prepared at a concentration of 10 mg/mL in methanol filtered using a 0.2μm Nalgene syringe filter (Thermo Scientific) and then diluted, to 1.25 mg/ mL, in 50% MeCN: %0% of 5% TFA prior to injection

Ten microliters of each sample was injected at a flow rate of 0.5mL/min, using a Symmetry C18 3.5 μm 4.6 x 150mm column with security guard C18 4mm x 2mm pre-column (Phenomenex) at room temperature. The method involved a gradient condition, starting at 65% mobile phase A (5% TFA in MilliQ water (w/w)), for 3

minutes; linear gradient to 85% mobile phase B (100% acetonitrile) between 3 and 12 minutes, with re-equilibration at 13 minutes. All analyses were performed on a Waters Alliance HPLC with detection by UV at 254nm (Fehr et al., 2009).

Statistical Analysis

For cell cytotoxicity assays, absorbance values were calculated and corrected for wells containing culture medium, no-extract controls and for blank wells containing PBS only. The percentage of growth inhibition was analysed with R , using the package drc (Ritz and Streibig, 2005) to fit 4 parameter logistic curves to the data with parameter values for the gradient, upper and lower asymptote and the EC_{50} , representing the endophyte extract concentration for half maximal inhibition. Analysis of variance was used to compare models fitting separate curves for each experiment with a single curve fitted to the combined data for each endophyte extract/cell line combination. Where separate curves needed to be fitted to the data, the mean EC_{50} value was the mean of the three curves (Table 2).

Results

Isolation and identification of the endophytic fungi

Seventeen isolates of endophytic fungi obtained from the leaves of *E. longifolia* (EL-1 – EL-17) were previously identified and described by Zaferanloo *et al*. (2013). The ITS regions of the ribosomal DNA from nine additional endophytic fungi isolated from *E. maculata* (EM-1 – EM-9) were amplified by PCR and sequenced. A BLASTN search of sequences available in the NCBI database yielded the identity of the isolates (Table 1). Morphological analyses supported identification obtained from the NCBI sequence data. Since the DNA sequences displayed significant similarities, it was suspected that the fungal isolates were evolutionarily close relatives and phylogenetic analysis was performed using DNA rather than protein sequences, and is summarised in Fig. 1, where bootstrap scores over 50 indicated that the sequences shared significant homology.

Cytotoxicity of endophyte extracts

The lyophilized preparations of all 26 endophyte fermentation broths were screened for anticancer/cytotoxicity activity. Three different human cancer cell lines (A549 (lung carcinoma); MOLT-4 (T-acute lymphoblastic leukaemia), PreB697 (PreB leukaemia) and a fibroblast cell line were incubated for three doubling-times with increasing concentrations of fungal endophyte extracts or pure AME or AOH. The *Alternaria* sp. extract, EM-6, was the most cytotoxic against MOLT-4 (EC_{50} 6.41 µg/mL) (Fig. 2A), whereas EM-7 and EM-9 were the most cytotoxic against PreB 697 (EC_{50} 39.7 µg/mL and 37.9 µg/mL respectively), (Fig. 2B and 2C). The only *Preussia* sp. endophyte extract that caused any cytotoxicity was EL-14: EC_{50} 116.0 µg/mL for MOLT4 and 42.10 µg/mL for PreB697 and 18.10 µg/mL for A549 (Fig. 2D). In all cancer cell lines, pure AOH or AME were substantially more toxic than the endophyte extracts (Fig. 3A and 3B). With respect to the normal fibroblast cells, only EM-6 (EC_{50} 46.6 µg/mL) and AOH were cytotoxic (EC_{50} 26.90 µg/mL) (Fig. 4).

UV-HPLC of cytotoxic endophyte extracts

To determine if the endophyte extracts contained AME or AOH and/or any novel metabolites, pure samples of AME, AOH and endophytic extracts were analysed using UV-HPLC. Chromatographic traces of endophytes were compared to pure AME and AOH traces for relative amounts of AME and AOH within each endophyte, but no specific quantification of AME or AOH within the extracts was attempted.

Retention times (RT) of AOH and AME were 11.2 and 13.9 minutes, respectively (Fig. 5). All cytotoxic extracts derived from *Alternaria* sp., EM-6, EM-7 and EM-9, had peaks corresponding to AME and AOH RT at varying area under the curve (AUC) concentrations whereas no such peaks were identified in the *Preussia* sp. Extract, EL-14 (Fig. 5). Three extra unique peaks were identified in EM6, two of which were also detected in the EL-14 chromatograph (Fig. 5)

Discussion

Endophytes from *E. longifolia* were successfully isolated and identified by Zaferanloo *et al*. (2013). Fourteen isolates were identified as belonging to eight different genera, including *Alternaria*, *Cladosporium*, *Fusarium*, *Leptosphaerulina*, *Nigrospora*, *Phoma*, *Preussia* and *Stemphylium*. These species have previously been identified as being endophytic to several plants (Márquez et al., 2012). The genera of the fungal isolates from *E. maculata* were obtained by sequencing of the ITS regions and were identified by a database search in GenBank with a match of at least 98% (E=0) (Table 1).

Isolate EM-1 was identified as a coelomycete, *Phoma* sp. *Phoma* spp. have previously been isolated and reported as fungal endophytes, with a high abundance in the roots and surrounding soil (Huang et al., 2009, Rodriguez et al., 2009, Chandra, 2012). Isolates EM-2 and EM-3 were identified as *Stemphylium* sp. Although this endophyte is not commonly cited in the literature, some studies demonstrated its ability to produce exopolysaccharide (Banerjee et al., 2009, Kharwar et al., 2011). EM-4 was identified as *Preussia* sp., a fungus previously reported as an endophyte (Kruys and Wedin, 2009). Isolate EM-5 was identified as *Chaetomium* sp. which has been frequently identified as a fungal endophyte with anticancer activity (Kharwar et al., 2011). Isolates EM-6, -7, -8 and -9 were identified as belonging to the genus *Alternaria* and shared significant homology. *Alternaria* spp. have been reported as known endophytes with antitumor activity in the native Australian plant, *Gossypium* (Wang et al., 2007, Chen et al., 2016); these plants are known to grow throughout similar regions of Australia where *E. maculata* is found. Notably, in this study, identification of the fungal isolates was performed by searching for matches in GenBank. It has been noted that identification based on this method relies on previous sequencing results deposited in the database to be accurate and taxonomic groups identified correctly (Bellemain et al., 2010). Furthermore, the non-coding ITS regions are subject to rapid evolutionary change, which creates additional difficulties in obtaining sequence matches and performing accurate sequence alignments (Huang et al., 2009). Thus, in this study, traditional morphological identification techniques were also used to support the molecular identification (Huang et al., 2009). It is further recommended that a range of different primers be used to amplify the ITS regions and analysing these alongside each other to generate a consensus as this would allow for more accurate reporting (Bellemain et al., 2010). Nevertheless, since the isolates in this study were identified with a

high percentage of similarity (Table 1) (Fig. 1), there is overall confidence in the identified genera. It is possible that other slow growing endophytes were not cultured from the plant samples and consequently did not undergo identification (Huang et al., 2009). Some fungi may not be cultivable on laboratory culture media and require conditions provided through an obligate host-symbiont relationship; these isolates may be identified by PCR analysis of total plant tissue derived DNA (Aly et al., 2011).

AME was not very toxic to the cell lines used in this study. Conversely, AOH had greater toxicity, particularly with respect to the MOLT4 and preB leukaemic cells. Of all the endophyte extracts tested, the most toxic was EM6 which appeared to have less AME and AOH than the other extracts but also had two unique peaks observed in the chromatograph (RT of 9.1 and 10.8 min.) and one peak at RT 14.5 min that was also present in EL-14. These extra metabolites may account for the increased toxicity of the EM6 extract towards MOLT4 and preB cells compared to the other endophyte extracts; however this would need further investigation. It was perhaps not surprising that AME and AOH were cytotoxic to these cancer cell lines to varying degrees but less toxic to the fibroblast cell line as they have previously been shown to be cytotoxic to other cancer cell lines (Aly et al., 2008).

The search for new anti-cancer agents continues as the number of affected people will continue to rise with increasing longevity. The discovery of new agents will improve the chances of prolonging life and curing more cancers. This study has demonstrated that Australian native plants are an untapped source of endophytic fungi that produce potentially useful medicinal compounds when cultured in the laboratory in the absence of the host plant. This will make it possible to produce medically-relevant metabolites without environmental impacts on plant populations and facilitate commercial production. Further work on these endophytic extracts is now required to identify and characterise the metabolites with anticancer potential and the development of methods to make such metabolites in commercial quantities is an important consideration.

Our team declare no conflict of interest in this study.

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Table 1: Identification of endophytic fungi based on the closest match of ITS sequences in the NCBI database[1]

Endophytes	Closest Species (accession number)	Identity (%)	Sequence length (bp)
EL-1	*Phomamoricola*(GQ352491.1)	98	545
EL-2	*Leptosphaerulinaamericana*(AY278318.1)	99	537
EL-3	*Stemphylium* sp. (EF104168.1)	100	456
EL-4	*Nigrospora* sp. (FJ785429.1)	99	483
EL-5	*Leptosphaerulinatrifolii*(AY831558.1)	99	538
EL-6	*Cladosporium* sp. (GU214631.1)	100	648
EL-7	Unclassified fungus (EU816402.1)	96	540
EL-8	*Phomaherbarum*(AB470824.1)	99	542
EL-9	*Phoma* sp. (AY210335.1)	98	493
EL-10	*Fusarium* sp. (DQ993637.1)	100	514
EL-11	*Leptosphaerulina* sp. (EF694653.1)	99	618
EL-12	*Alternaria* sp. (GQ302684.1)	99	570
EL-13	Unclassified fungus (EF419981.1)	97	574
EL-14	*Preussia minima* (DQ468035.1)	95	474
EL-15	*Alternaria* sp. (GU187964.1)	100	607
EL-16	Unclassified fungus (Ef420002.1)	97	544
EL-17	*Alternaria alternata* (FJ375168.1)	100	506
EM-1	*Phoma* sp. (GQ352491)	98	545
EM-2	*Stemphylium* sp. (EF104156)	100	554
EM-3	*Stemphylium* sp. (EF076750)	100	500
EM-4	*Preussia* sp. (HQ130664)	99	482
EM-5	*Chaetomium* sp. (GU934507)	99	519
EM-6	*Alternaria* sp. (HQ914864)	99	524
EM-7	*Alternaria* sp. (HQ914865)	100	522
EM-8	*Alternaria* sp. (HQ914850)	99	522
EM-9	*Alternaria* sp. (HQ914865)	99	519

[1]Sequences have been submitted to the GenBank database and assigned the accession numbers JQ316434–JQ316450 for EL samples and JX262252-JX262260 for EM samples.

Table 2: IC50 values (µg/ml; standard errors in parentheses) for Alternariol (AOH), alternariol methyl ester (AME) and extracts EL14, EM6, EM7 and EM9 from the cytotoxicity curves. Data were derived from the mean of three experiments (except n=1 for fibroblasts); ud, IC50 undefined due to lack of statistical significance (not significantly different from 0).

	AME	**AOH**	**EL14**	**EM6**	**EM7**	**EM9**
Molt4	ud	19.10 (1.90)	116.00 (32.2)	6.41 (1.97)	61.60 (3.62)	21.20 (1.95)
A549	ud	76.50 (25.5)	ud	35.40 (11.0)	89.70 (50.4)	132.00 (23.9)
preB	27.00 (7.88)	39.40 (7.02)	42.10 (14.3)	4.28 (0.89)	39.70 (10.3)	ud
Fibro	ud	ud	ud	46.60 (25.0)	ud	ud

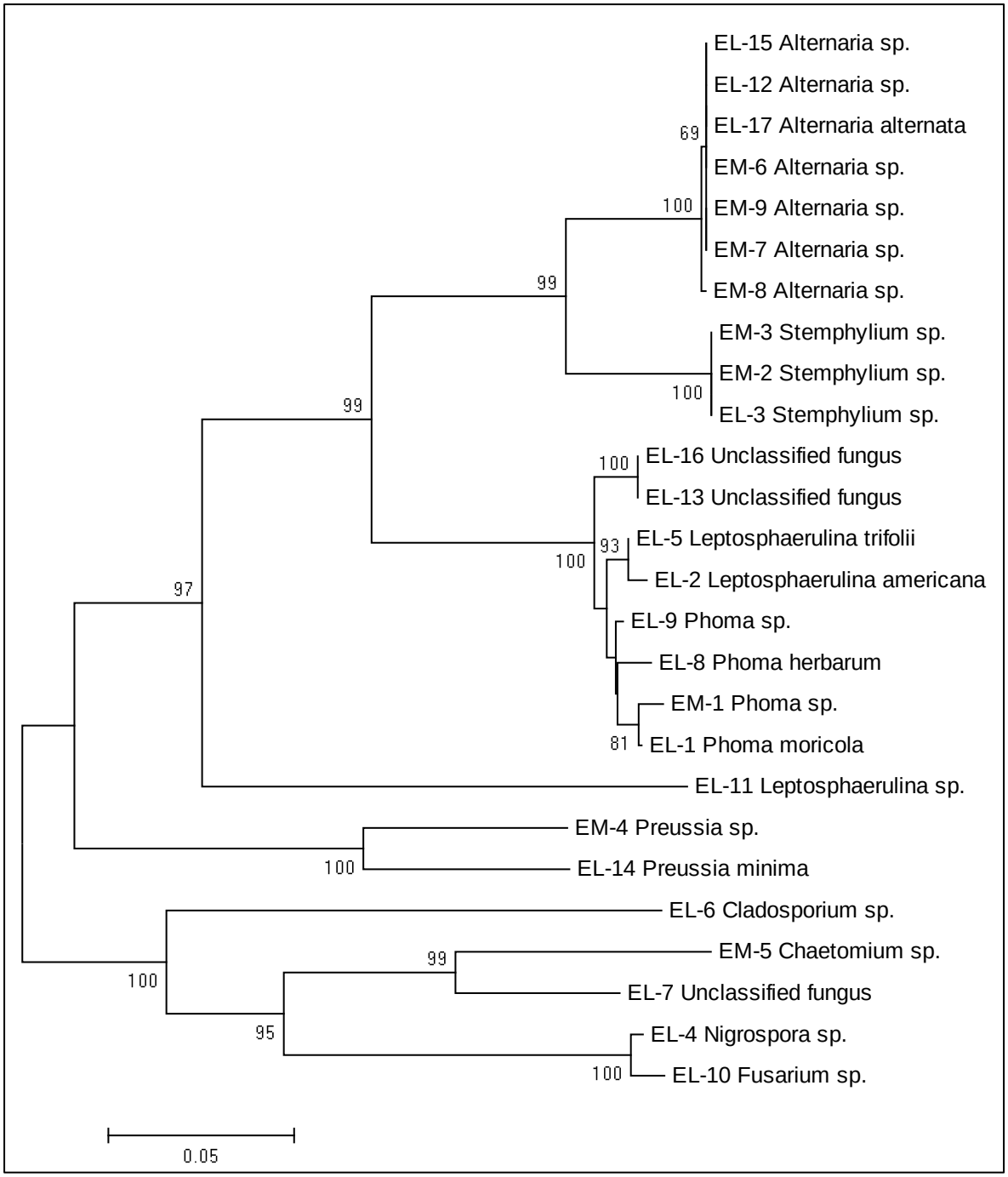

Figure 1: The ITS regions of fungal endophytes were used to construct a phylogenetic tree of the isolates obtained. The Neighbour-Joining method was used to infer phylogeny using MEGA version 5; p-distance and a bootstrapping score of 1000 with values of greater than 50 % were employed parameters. Significant homology that suggests these isolates could belong to the same species or even subspecies. (Kumar et al., 2016).

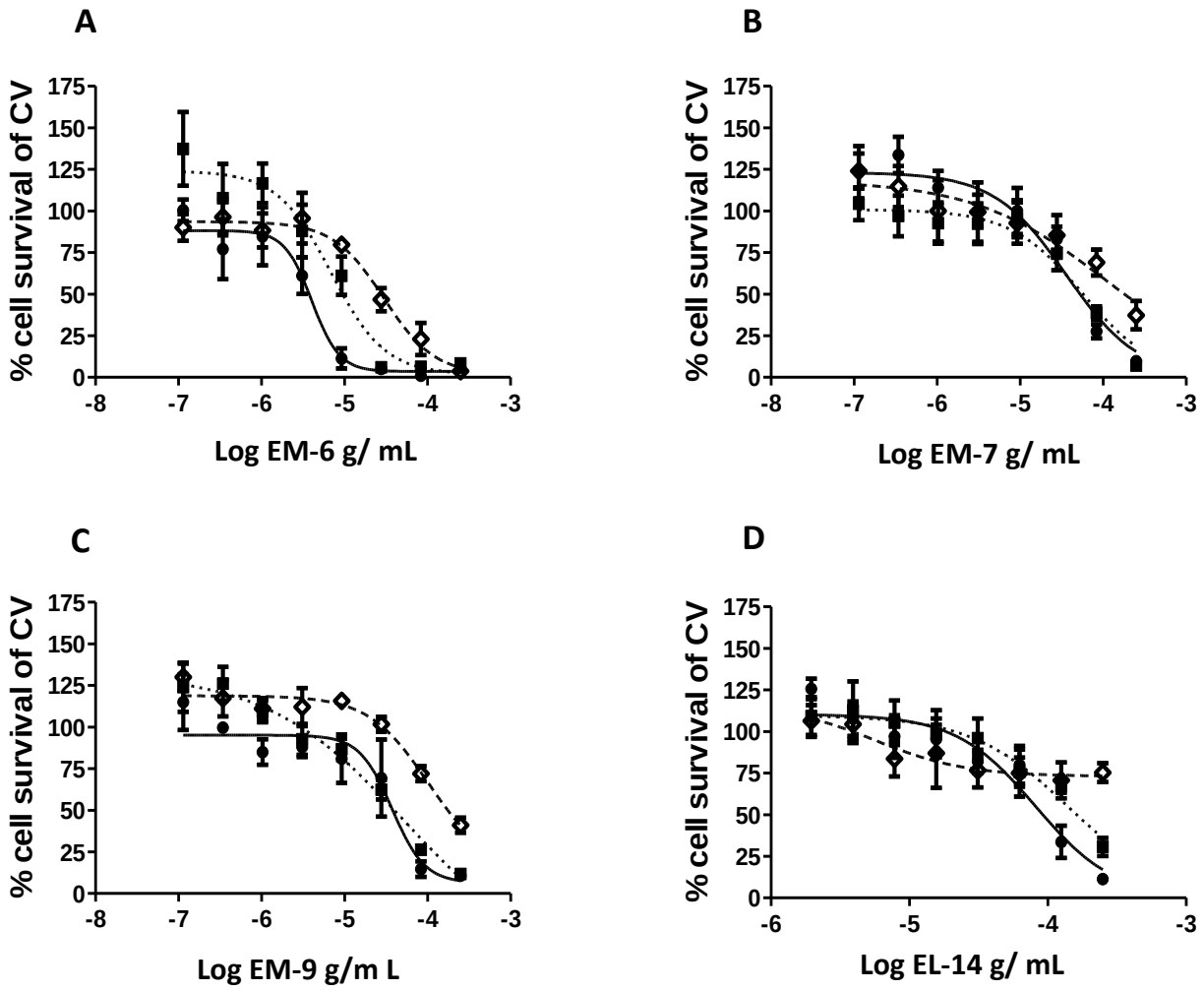

Figure 2: Human cancer cell line cytotoxicity MOLT-4▪, PreB697• and A549 ◊ to endophytes **A)** EM-6 , **B)** EM-7, **C)** EM-9 and **D)** EL-14. Error bars represent the S.D. of the means from three separate experiments.

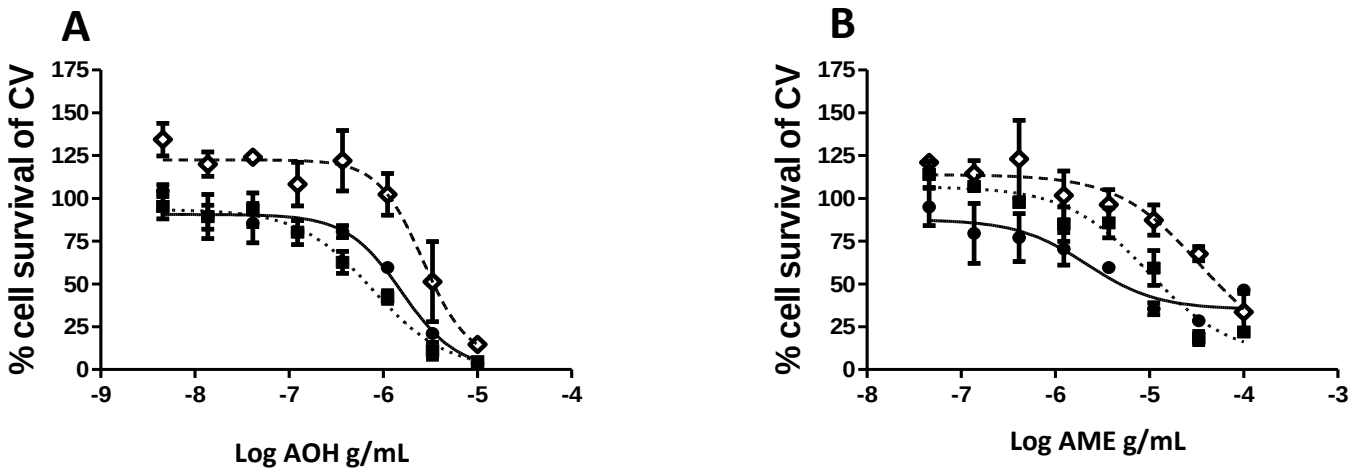

Figure 3: Human cancer cell line cytotoxicity MOLT-4 ▪ PreB697 • and A549◊ to **A)** Alternariol monomethyl ether (AME) and **B)** Alternariol (AOH). Error bars represent the S.D. of the means from three separate experiments.

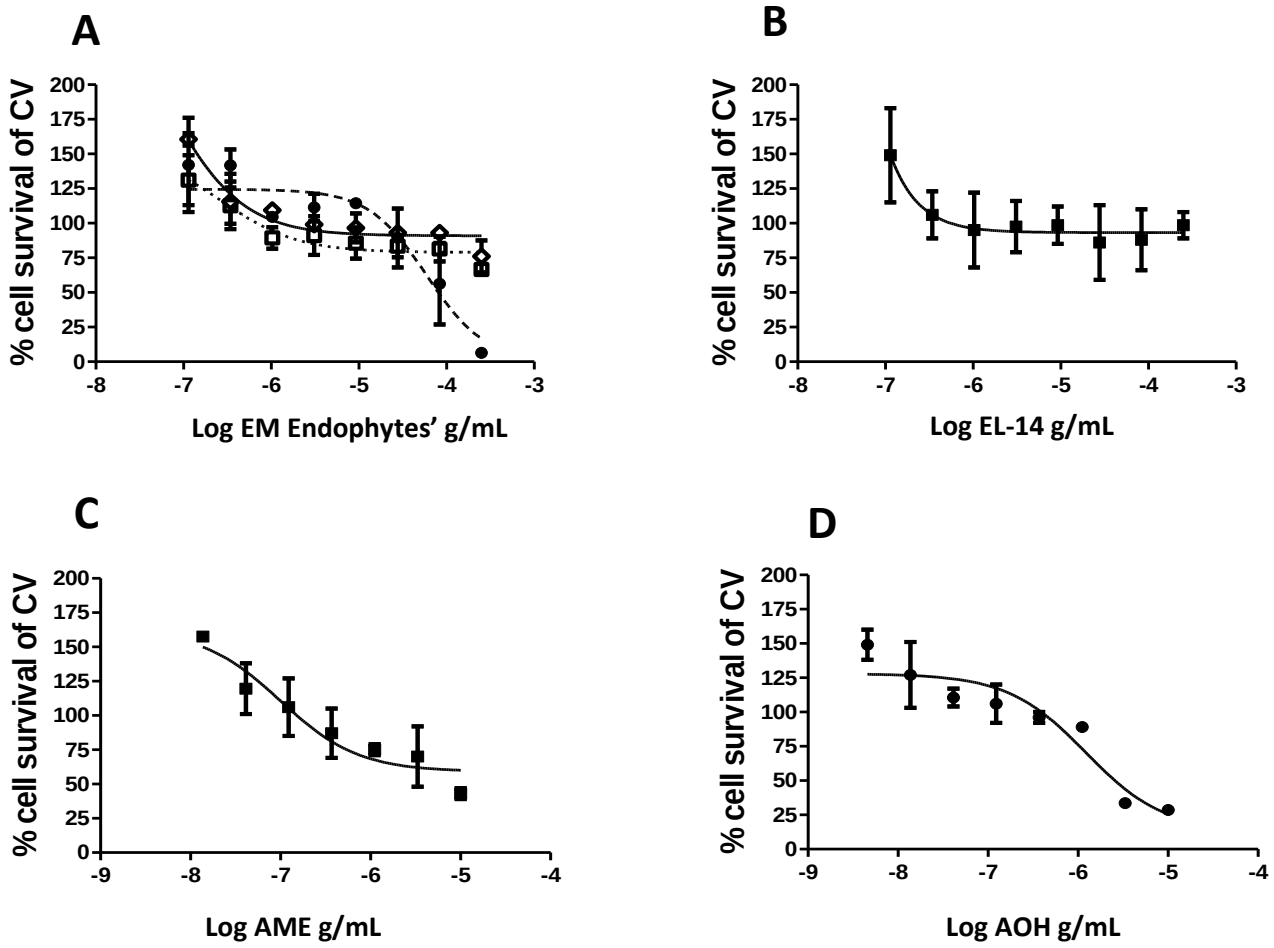

Figure 4: Human fibroblast cell line cytotoxicity to endophytes **A)** EM-6 •, EM-7 □, EM-9 ◊; **B)** EL-14 ▪; **C)** Alternariol monomethyl ether (AME) and **D)** Alternariol (AOH). Error bars represent the standard error of the means from three separate experiments.

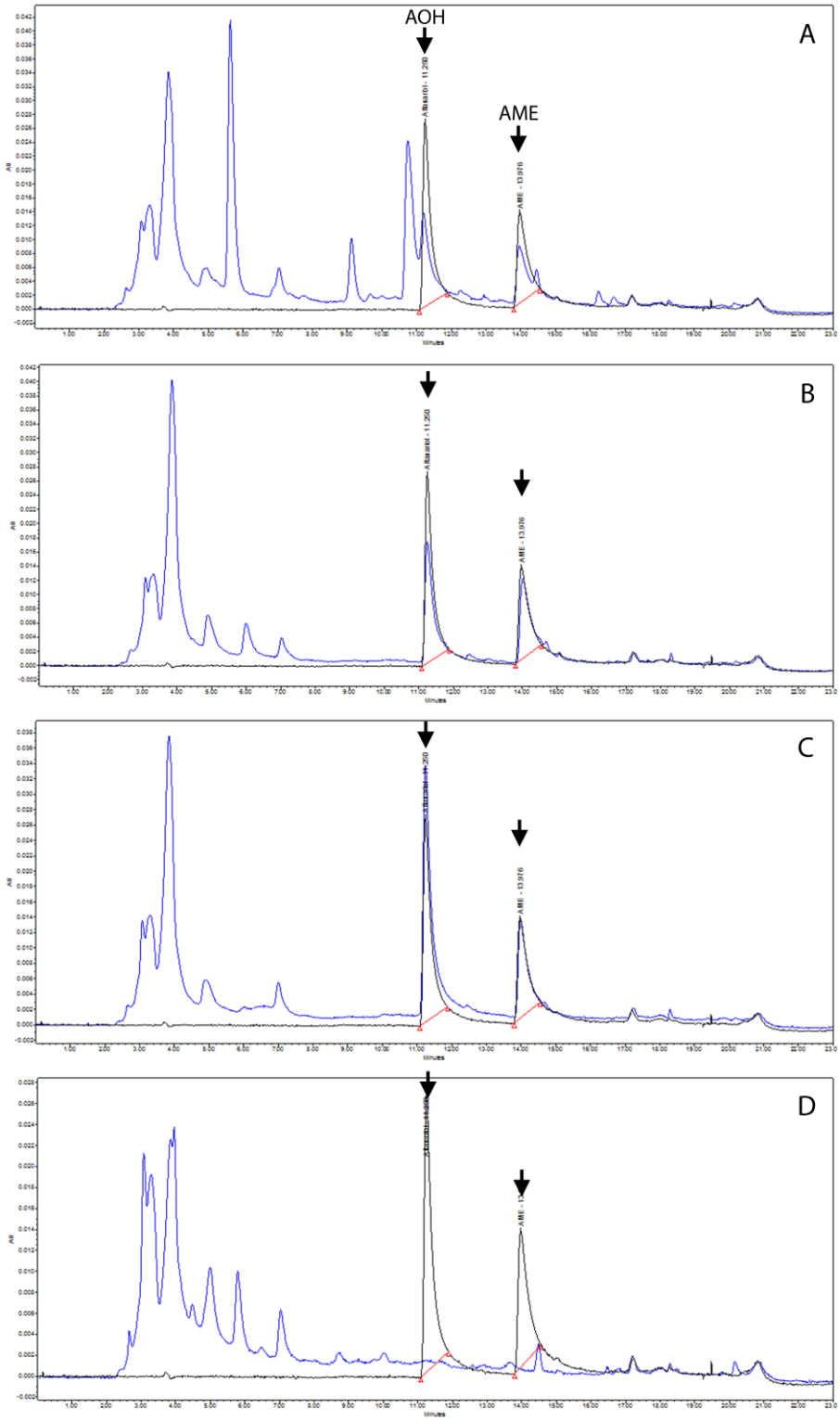

Figure 5: HPLC trace of endophytes **A)** EM-6, **B)** EM-7, **C)** EM-9, **D)** EL-14 in blue lines and standards alternariol monomethyl ether (AME) with retention time 13.9 minutes and alternariol (AOH) with retention time 11.2 minutes superimposed in black showing the presence of AME and AOH in EM-6, EM-7 and EM-9 and unidentified peaks in EM-6 at retention times of 9.1, 10.8 and 14.5 minutes and EL-14 at retention time of 14.5 minutes